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GLUCOCORTICOIDS SUPPRESS CALCIUM MOBILIZATION AND PHOSPHOLIPID HYDROLYSIS IN ANTI-Ig ANTIBODY-STIMULATED B CELLS[1]

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Glucocorticoids have been shown to play a major role in influencing the activation of B lymphocytes. In view of our recent observation that dexamethasone exerts a marked suppressive effect on an early event in B cell activation that is stimulated by anti-Ig antibody, we investigated its activity on other stimuli that induce intracellular events similar to those produced by anti-Ig antibody. Because the intracellular events that occur after B cell stimulation with phorbol myristate acetate and the calcium ionophore A23187 appear to mimic those that occur after B cell stimulation with anti-Ig antibody, we studied whether the cellular responses elicited by these activation stimuli are affected in a similar fashion by dexamethasone. Whereas anti-Ig antibody-stimulated entry of G_0 B cells to the G_1 and S phase of the cell cycle was markedly suppressed by dexamethasone, phorbol myristate acetate/A23187 stimulation of these events was resistant to dexamethasone. Our finding that anti-Ig-induced cross-linking of B cell surface Ig, as measured by surface Ig capping, was not inhibited by dexamethasone suggested that corticosteroids inhibit anti-Ig-induced B cell proliferation at a step distal to membrane Ig cross-linking and proximal to phosphatidylinositol bisphosphate hydrolysis. This hypothesis is supported by experiments presented in this manuscript which demonstrate that dexamethasone inhibits anti-Ig-stimulated phosphatidylinositol bisphosphate hydrolysis. We also found that dexamethasone markedly inhibited anti-Ig antibody-stimulated increases in intracellular ionized calcium concentrations. This dexamethasone-mediated suppression is time-dependent as it is not seen when B cells are cultured with dexamethasone for less than 6 hr. Our data suggest that the immunomodulatory activity of glucocorticoids is exerted by binding to its nuclear receptor, thereby preventing the generation of second messengers required for cell activation after agonist-receptor interaction.

Many mitogenic stimuli utilize a pathway of cellular activation that results in an increase in the functional activity of protein kinase C (PKC),[3] an enzyme which has been implicated in regulation of cellular proliferation (1, 2). In B cells, anti-Ig antibody-induced cross-linking of membrane surface Ig (sIg) stimulates the activation of PKC and the release of intracellular calcium as a consequence of the phospholipase C-mediated hydrolysis of membrane phospholipids and generation of diacylglycerol and inositol trisphosphate (3, 4).

The plasma membrane signals preceding PKC activation can be bypassed in vitro by stimulation of B cells with a combination of phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (5–9). As such, the synergism that has been observed between PMA and calcium ionophore for B cell activation is considered to be a model for anti-Ig-induced B cell activation. If however, this is to be utilized as an ideal model, then the B cell responses elicited by the synergistic combination of PMA and calcium ionophore should not only mimic those elicited by anti-Ig, but should also be regulated similarly. We have therefore investigated various aspects of B cell proliferation that are induced both by anti-Ig antibody and by PMA/A23187. Because we have recently demonstrated that glucocorticoids suppress the expression of B cell sIa (10), a relatively early marker of B cell activation, we thought dexamethasone would be a useful probe to compare early events in B cell activation stimulated by anti-Ig or PMA/A23187. The present findings demonstrate the marked dexamethasone susceptibility of the anti-Ig antibody-stimulated B cell proliferative response in contrast to the resistance of the PMA/A23187-stimulated B cell proliferative response. The findings that anti-Ig-induced cross-linking of B cell surface Ig and induction of cap formation occur normally in the presence of dexamethasone suggested to us that glucocorticoids may exert their inhibitory effects at a point distal to membrane Ig cross-linking but before PKC activation. In support of

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[3] Abbreviations used in this paper: PKC, protein kinase C; LPS, lipopolysaccharide; $[Ca^{2+}]_i$, ionized calcium; PMA, phorbol 12-myristate and 13-acetate; NK, natural killer; [^{3}H]Thd, [^{3}H]thymidine; PIP_2, phosphatidylinositol bisphosphate; BSF-1, B cell stimulatory factor 1.

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this hypothesis we have found that dexamethasone suppressed anti-Ig-stimulated phosphatidylinositol turnover as well as increases in intracellular ionized calcium concentration, $[Ca^{2+}]_i$. These experiments suggest that glucocorticoids interfere with early events in cell activation by inhibiting the generation of second (inositol trisphosphate) and third $[Ca^{2+}]$ messengers.

MATERIALS AND METHODS

Animals. DBA/2 and BALB/c female mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were used at 8 to 12 weeks of age.

B cell purification. Spleens were teased apart in a petri dish containing Hank's balanced salt solution, filtered through gauze mesh, washed, and resuspended in RPMI 1640 containing 10% fetal bovine serum (B cell media). B cells were enriched by a modification of the method of Liebson et al. (11). In some experiments, B cell preparations were depleted of natural killer cells using rabbit anti-asialo-GM_1 antibody (Wako Pure Chemical Industries, Dallas, Texas) (12). The purified B cells obtained by this method contained less than 1% T cells as measured by fluorescence-activated cell sorter analysis of fluorescein anti-Thy-1.2 stained cells. In addition, these cells did not proliferate in response to the T cell mitogen concanavalin A after 2 and 3 days of in vitro culture.

Enrichment for resting B cells. Fractionation of B cells into size-dependent subpopulations was done by Percoll density centrifugation in most experiments using Percoll concentrations of 50, 60, 65, and 70%. Purified B cells (1×10^8/ml) were overlayed on a total of 10 ml of Percoll, incubated for 15 min at 4°C, and then centrifuged at 2300 × G for 15 min. The 70% fraction was recovered (small resting B cells) and suspended in culture medium at the appropriate cell concentration for addition to culture dishes. In other experiments, purified B cells were separated into five size-dependent B cell subpopulations by counter flow centrifugation (Beckman centrifuge 1218 with elutriation rotor JE6; Beckman Instruments, Palo Alto, CA) using a standard cell separation chamber (No. 354690, Beckman Instruments). Cell suspensions were diluted into 50 ml of high phosphate-buffered saline. After centrifugation at 250 × G for 10 min, the cells were resuspended in elutriation buffer (RPMI containing 1% fetal calf serum and 100 mM EDTA) and fractionated as previously described (13).

Cell sizing and counting. Cells were counted and sized on a linear scale using a Coulter ZB counter (Coulter Electronics, Hialeah, FL) equipped with a Coulter Accucomp/C-1000 Cell Sizer with a 100 μ^3m aperture.

Measurement of inositol phosphate accumulation and $[Ca^{2+}]_i$. Accumulation of inositol phosphates was measured by a modification of the procedures of Berridge as described by Beaven et al. (14). The procedure for the measurement of $[Ca^{2+}]_i$ in single cells has been described elsewhere (15). Briefly, cells were loaded with the acetoxymethylester of indo-1 (Molecular Probes, Junction City, OR) by using an initial concentration of approximately 2.0 μM. After the loading procedure, cells were washed, placed in fresh medium at 2.5×10^6/ml, and generally stored in the dark at room temperature until analysis. Immediately before each assay, indo-1-loaded cells were diluted to 1×10^6/ml with medium, equilibrated at 37°C, and analyzed by flow cytometry at 400 to 500 cells/sec. For each cell analyzed, the ratio of violet to blue fluorescence was digitally calculated in real time and multiplied by a factor of 5 in order to scale the data to the display. The $[Ca^{2+}]_i$ (nanomolar) can be calculated from the indo-1 violet to blue ratio using the formula derived by Grynkiewicz et al. (16) so that a flow cytometric ratio normalized to 1 for resting T lymphocytes corresponds to a $[Ca^{2+}]_i$ of approximately 130 nM, a ratio of 2.0 = 338 nM, and a ratio of 3.0 = 678 nM (15). We found that the basal $[Ca^{2+}]_i$ of murine B cells was not detectably different from human T cells. In practice, before each assay, small, resting B cells were used to establish a ratio of violet to blue fluorescence of 1.0, and calcium ionophore-treated cells were analyzed to confirm that the ratio of R_{max} to R was approximately 6.5. There are 100 data points on the x (time)-axis on plots of either mean indo-1 ratio or percent responding cells. Each of these data points represents the average value of approximately 2000 cells analyzed during the 6 sec comprising each data point, and therefore, intra-assay estimates of error about the mean are not displayed. This technique is more sensitive than the quin2 assay as there is a 6- to 7-fold increase in the indo-1 fluorescence ratio after calcium ionophore stimulation of quiescent cells, whereas there is only an approximate 2-fold increase in the fluorescence intensity of quin2-loaded cells (15). Furthermore, cells are loaded with 10- to 20-fold lower concentrations of the fluorescent indicator, therefore minimizing the potential effects of chelation of free calcium by the indicator. In addition, the technique is capable of detecting a calcium response in as few as 0.3% of cells analyzed. Potential limitations of the assay are that the indo-1 esters are assumed to be completely hydrolyzed and that indo-1 is assumed to be evenly distributed throughout nuclear and cytoplasmic compartments. Under some conditions, however, indo-1 is incompletely hydrolyzed (17) and can become compartmentalized, thus invalidating conventional calibration techniques and potentially, permitting regional buffering of calcium transients. The ratios of calcium sensitive violet to blue indo-1 fluorescence shifts are decreased by residual indo-1 esters, which exhibit UV-excited, calcium-insensitive fluorescence primarily at blue wavelengths. We found that murine B cells did not contain significant residual indo-1 ester, as the observed fluorescence shifts of calcium ionophore-treated cells closely approximated those predicted from the spectral properties of indo-1 in a buffer resembling intracellular conditions (not shown).

Cell culture and harvest. For evaluation of B cell DNA synthesis, 1×10^5 cells/well were cultured in flat-bottomed microtiter plates (Costar, Cambridge, MA) in a total volume of 0.2 ml in modified Mishell Dutton medium that contained 10% endotoxin-free fetal bovine serum (Hyclone, Logan, UT) and 5×10^5 M 2-mercaptoethanol. Experimental samples performed in triplicate were incubated in a humidified 5% CO_2 incubator. Cultures were pulsed with 1.0 <Ci of [*methyl*-^{3}H-thymidine] ([^{3}H]Thd) 18 hr before harvesting. The results are expressed as the arithmetic mean ± SD of triplicate determinations in counts per minute per culture.

Analysis of B cell DNA content. The percentage of B cells in S, G_2, and M phases of the cell cycle was measured by a determination of propidium iodide binding. Propidium iodide, 50 μg/ml (Sigma Chemical Co., St. Louis, MO), dissolved in 0.5% Nonidet P-40 in 1.12% citrate buffer, pH 8.4, was used to stain cells as previously described (18), followed by analysis on a fluorescence-activated cell sorter. The percentage of cells in S, G_2, and M phases of the cell cycle was determined by measuring the integral of cells with greater than 2 N DNA content.

Reagents. Affinity-purified goat antibody to mouse sIgD was prepared as previously described (18). Lipopolysaccharide W (LPS) *Escherichia coli* 0111:B4 was purchased from Difco Laboratories (Detroit, MI). Phorbol 12-myristate 13-acetate and the calcium ionophore, A23187, were purchased from Sigma. PMA and A23187 was dissolved in ethanol and dimethyl sulfoxide, respectively, and stored at −80°C. B cell stimulatory factor 1 (BSF-1) was purified as described by Ohara et al. (19). Dexamethasone sodium phosphate was generously provided by Dr. Clement A. Stone (Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ).

RESULTS

Dexamethasone suppression of B cell proliferation. In previous experiments we have demonstrated that corticosteroids suppress both the expression of B cell surface Ia (10) as well as anti-Ig-stimulated B cell proliferation (unpublished observations). To study the mechanism by which corticosteroids suppress anti-Ig-induced B cell activation we investigated the ability of dexamethasone to suppress B cell proliferation stimulated by PMA and A23187, agents which bypass surface receptors and mimic the intracellular events that occur during anti-Ig stimulation (Table I). When dexamethasone was added at the initiation of the culture period, it dramatically inhibited anti-Ig antibody-stimulated B cell DNA synthesis, whereas LPS-stimulated B cell proliferation was affected to a significantly lesser extent. In contrast, B cell stimulation by PMA and A23187 was affected minimally, if at all. These findings indicated that B cell proliferation that was stimulated by the synergistic combination of PMA and A23187 was more resistant to the suppressive influence of dexamethasone whereas that stimulated by anti-Ig was dexamethasone-sensitive. This observation suggested that dexamethasone was exerting its suppressive effect on anti-Ig-induced B cell activation at some activational step proximal to the activation of PKC and induction of calcium translocation, which are direct effects of PMA and A23187. In view of the possibility that costimulation with PMA/A23187 was activating B cells

TABLE I

Dexamethasone induced suppression of B cell proliferation stimulated by anti-Ig antibody but not by PMA and A23187[a]

	[^{3}H]Thd Incorporation (cpm)		
	Medium	Dex 10 nM	Dex 100 nM
Medium	7,749	2,208	593
BSF-1	9,223	3,432	1,097
Anti-IgD	43,690	7,573	2,151
Anti-IgD + BSF-1	57,738	14,373	2,572
PMA	3,013	1,407	523
A23187	6,417	1,621	577
PMA + A23187	69,887	64,050	59,579
LPS	78,074	60,889	38,195

[a] Resting B cells (2×10^5 cells/well) were cultured in the presence of: BSF-1, 10 U/ml; anti-IgD, 10 μg/ml; PMA, 10 ng/ml; A23187, 250 ng/ml; or LPS, 50 μg/ml, for 48 hr. Cells were pulsed with 1 μCi of [^{3}H]Thd 24 hr before harvesting. Dexamethasone (Dex) was added at the initiation of the culture. The values shown represent the arithmetic mean of triplicate determinations. All SD were less than 10% of the mean.

TABLE II

Effects of delayed administration of dexamethasone on anti-Ig-activated B cells[a]

	[^{3}H]Thd Incorporation (cpm)		
	Medium	Dex 10 nM	Dex 100 nM
Medium	7,167	4,075	1,994
BSF-1	11,113	5,265	3,138
Anti-IgD	41,013	22,303	10,402
Anti-IgD + BSF-1	65,609	37,063	14,682
PMA	4,301	1,627	1,622
A23187	8,871	3,420	1,683
PMA + A23187	88,446	71,424	84,037
LPS	80,504	74,447	52,677

[a] Resting B cells (2×10^5 cells/well) were cultured in the presence of: BSF-1, 10 U/ml; anti-IgD, 10 μg/ml; PMA, 10 ng/ml; A23187, 250 ng/ml; or LPS, 50 μg/ml, for 48 hr. Cells were pulsed with 1 μCi of [^{3}H]Thd 24 hr before harvesting. Dexamethasone (Dex) was added 24 hr after the initiation of the cultures. Table I shows the responses of stimulated cells in the continuous presence of dexamethasone using the same starting B cell preparation as was used in this experiment. The values shown represent the arithmetic mean of triplicate determinations. All SD were less than 10% of the mean.

more rapidly to a stage at which they were less susceptible to the suppressive effect of dexamethasone than was anti-Ig-induced activation, we studied the effect of dexamethasone on B cell proliferation when it was added 24 hr after the initiation of the cell cultures (Table II). Even when administered after B cells have been stimulated for 24 hr by anti-Ig or the combination of anti-Ig and BSF-1, dexamethasone was inhibitory, albeit not to the same extent as when it was added at the initiation of the culture period; in this same experiment responses stimulated by PMA/A23187 were resistant to the dexamethasone-mediated suppressive effects. This result suggests that anti-Ig-stimulated cells maintain their sensitivity to dexamethasone even after they have been activated to G_1 of the cell cycle.

We have in an occasional experiment observed that dexamethasone can suppress PMA/A23187-stimulated B cell proliferation by 20 to 30% as compared to controls cultured in the absence of dexamethasone. Because it was possible that the occasional dexamethasone-mediated suppression of PMA/A23187 stimulation might have reflected the presence of suboptimal intracellular concentrations of mediators generated by these agents thus resulting in a less potent stimulatory signal, we studied the effect of dexamethasone on B cell DNA synthesis induced by multiple dose combinations of PMA/A23187 (Table III). In addition, because we had also observed differences in the magnitude of the response when different murine strains were used, we studied the B cell response in BALB/c mice as well as in DBA/2 mice (Table III). Proliferative responses of B cells derived from BALB/c spleen were consistently lower than the proliferative responses of B cells from DBA/2 spleen. Significant B cell stimulation was achieved in both murine strains with concentrations of PMA at 1 ng/ml and with concentrations of A23187 as low as 160 ng/ml. With the higher PMA concentration (10 ng/ml), B cell proliferation occurred with A23187 at 120 ng/ml. This very narrow dose-response titration of A23187 might explain the inability of other investigators to observe B cell proliferation stimulated by PMA/A23187 (6–8). Even at the threshold combinations of PMA/A23187 the proliferative response of BALB/c-derived B cells was unaffected by dexamethasone, while the stimulation of B cells derived from DBA/2 was marginally suppressed by dexamethasone at the lower concentrations of A23187 used. This suppressive effect of dexamethasone on the PMA/A23187-stimulated proliferative response was observed in approximately 20% of the experiments of this design. It is not clear to us whether this represents an actual suppression in view of the marked suppressive effects that dexamethasone exerts on cultures of unstimulated B cells ("background" counts per minute) and thus the calculated stimulation index of B cell proliferation stimulated by PMA/A23187 is not diminished even in the presence of dexamethasone. Likewise, the significance of murine strain variation of this response is unknown and will require additional investigation. The marked inhibitory effect, however, of dexamethasone on anti-Ig-stimulated responses was always reproducible in all strains of mice that were tested.

Dexamethasone suppresses cell volume increases of anti-Ig-stimulated B cells, but not of PMA/A23187-stimulated B cells. Increases in B cell volume that occur in response to mitogenic stimulation correlate with entry

TABLE III

Dexamethasone suppression of B cell proliferation: effect on B cells stimulated by multiple synergistic dose combinations of PMA and A23187[a]

	[^{3}H]Thd Incorporation (cpm)			
	BALB/c		DBA/2	
	Medium	Dex 100 nM	Medium	Dex 100 nM
Medium	1,235	586	2,503	562
BSF-1	2,356	625	4,111	1,043
Anti-IgD	2,305	942	10,442	2,451
Anti-IgD + BSF-1	13,452	1,839	84,017	6,450
LPS	42,882	15,533	117,989	49,533
Concanavalin A	1,189		2,056	
PMA (1 ng/ml)	437	455	730	775
+ A23187 (200 ng/ml)	7,664	11,827	22,658	20,980
+ A23187 (160 ng/ml)	9,911	8,960	9,529	5,381
+ A23187 (120 ng/ml)	1,826	2,333	1,293	753
+ A23187 (80 ng/ml)	424	527	485	562
PMA (10 ng/ml)	1,004	577	1,637	807
+ A23187 (200 ng/ml)	13,323	17,381	55,023	56,834
+ A23187 (160 ng/ml)	15,305	17,959	47,853	30,100
+ A23187 (120 ng/ml)	7,461	8,815	ND[b]	ND
+ A23187 (80 ng/ml)	1,621	924	ND	ND

[a] Small resting B cells (1×10^5/well) were cultured in the presence of B cell stimuli: BSF-1, 10 U/ml; anti-IgD, 10 μg/ml; LPS, 50 μg/ml; concanavalin A, 2 μg/ml; and the indicated concentrations of PMA and A23187 for 48 hr. Cells were pulsed with 1 μCi of [^{3}H]Thd 18 hr before analysis. Numerical values represent the arithmetic mean of triplicate determinations. [^{3}H]Thd uptakes of cells in the presence of A23187 doses alone (not shown) were all less than control values with exception of the following doses: DBA/2, 200 (794), 80 (570), and BALB/c, 120 (604), all of which were in the presence of and not significantly different from that with dexamethasone (Dex) 100 nM alone. All SD were less than 10% of the mean.

[b] ND, not determined.

of B cells into G_1 phase of the cell cycle. In order to determine at which stage of B cell activation dexamethasone is exerting its effect, we investigated cell volume increases in response to anti-Ig or PMA/A23187 in its presence or absence (Fig. 1). In the presence of dexamethasone, anti-Ig was not able to elicit increases in B cell volume, whereas volume increases induced by PMA/A23187 were unaffected. In addition, incubation of B cells with dexamethasone for as few as 6 hr, followed by extensive washing and an additional 18-hr culture period in medium alone before stimulation with anti-Ig, resulted in significant inhibition of cell volume increases (data not shown). This finding indicates that dexamethasone prevents anti-Ig-stimulated B cells from entering G_1 phase of the cell cycle, but does not prevent PMA/A23187-stimulated B cell activation.

Suppressive effect of dexamethasone on B cell proliferation is mediated by binding to its nuclear receptor. Recently a number of investigators have demonstrated that a compound termed RU486 which inhibits binding of progesterone to its cellular receptor also blocks (but with lower avidity) the binding of cortisol to its nuclear receptor (20). To demonstrate that the dexamethasone-mediated suppressive effects on B cell activation were mediated by its binding to its nuclear receptor, we cultured cells with dexamethasone in the presence or absence of RU486. Cells that were cultured with RU486 showed responses that were not significantly different from those of cells incubated in medium alone (data not shown). However, while dexamethasone exerted a dramatic suppressive effect on anti-Ig and LPS-stimulated B cell proliferation, it was significantly less inhibitory when cultured in the presence of RU486 (Table IV). Because RU486 is not 100% efficient in blocking the binding of cortisol to its receptor, it is not surprising that even in the presence of RU486 some suppressive effects of dexamethasone on B cell proliferation were noted. These results indicate that it is the dexamethasone binding to its cellular receptor that mediates the B cell-suppressive effects.

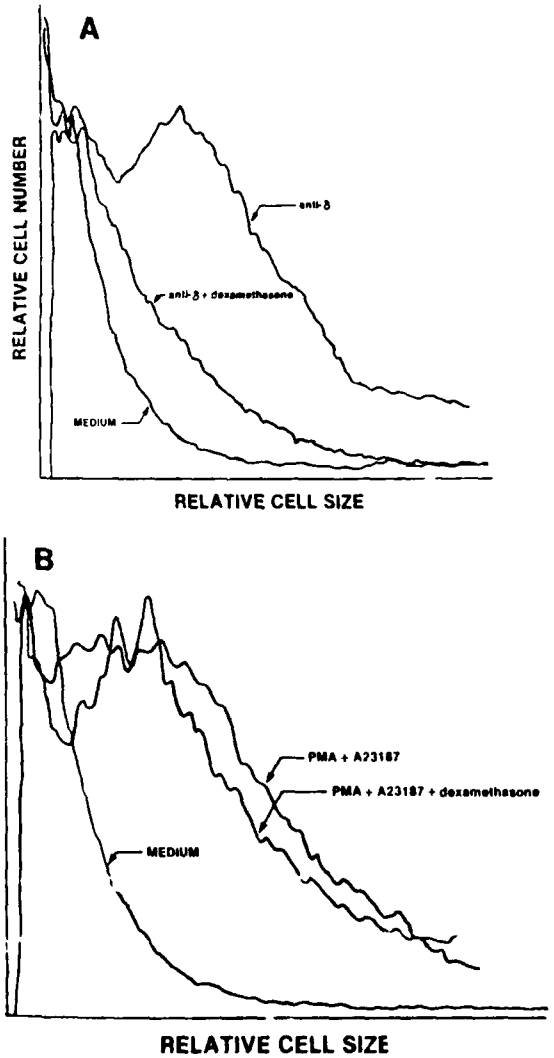

Figure 1. Percoll fractionated DBA/2 B cells were cultured for 18 hr with PMA (100 ng/ml) + A23187 (200 ng/ml) or anti-IgD (10 μg/ml) in the presence or absence of dexamethasone (100 nM) and sized on a Coulter channelizer after removal of nonviable cells as described under *Materials and Methods*.

Inability of dexamethasone to suppress PMA/A23187-stimulated B cell proliferation does not reflect PMA/A23187 stimulation of sIg⁻ cells in culture. In view of the finding that dexamethasone suppressed B cell proliferation stimulated by soluble anti-Ig but not that stimulated by PMA/A23187 it was critical to demonstrate that PMA/A23187 activation of B cells was not influenced by the presence of other non-B cells. Because natural killer (NK) cells and macrophages can be activated by the combination of PMA/A23187, they could potentially contribute to overcoming the suppressive effect exerted by dexamethasone on B cell proliferation. We therefore prepared T cell- and NK cell-depleted B cell populations, followed by differential centrifugation to enrich for the smallest sized B cell fraction. This fraction exhibited no T cell or NK cell activity, had no detectable esterase-positive cells and showed excellent proliferative response to the combination of PMA/A23187. This observation supports the findings of others (7, 9) and suggests that PMA/A23187 is directly activating B cells to synthesize DNA and is not stimulating DNA synthesis in B cells indirectly via the stimulation of lymphokine secretion from NK cells, T cells, or macrophages. Importantly dexamethasone suppressed the anti-Ig-stimulated but not PMA/A23187-stimulated B cell proliferative response in this cell population.

Although we had excluded the possibility that significant numbers of non-B cells could be participating in the

TABLE IV

Inhibitory effect of dexamethasone on B cell activation is mediated by its binding to its nuclear receptor[a]

Mitogen	Medium	[³H]Thd incorporation (cpm)	
		Dexamethasone	Dexamethasone + RU486
Medium	6,110	363	6,998
Anti-IgD + BSF-1	18,345	3,710	11,795
LPS	65,027	22,445	49,376

[a] Percoll-fractionated B cells were cultured at 1×10^5 cells/well in the presence of medium, anti-IgD (100 μg/ml) + BSF-1 (10 U/ml), or LPS (50 μg/ml). Dexamethasone (10^{-8} M) or RU486 (10^{-7} M) was added 30 min before the addition of mitogens. At 36 hr 1.0 μCi of [³H]Thd was added and cells were harvested 18 hr thereafter. The results represent the arithmetic mean of triplicate cells. Cells that were cultured with RU486 in the presence of mitogens mounted a proliferative response that was not significantly different from cells cultured with medium (data not shown).

responses that we observed to PMA/A23187, the possibility still remained that PMA and A23187 acted synergistically to stimulate extremely small numbers of potentially contaminating non-B cells in our B cell population to produce factors that might influence B cell DNA synthesis. At more limiting B cell densities of 5×10^4 cells/well and lower, the contribution of such non-B cell-derived soluble factors to B cell stimulation would more likely become apparent. This would result in a nonlinear decline in the B cell proliferative response with decreasing concentrations of cells cultured. We therefore studied the effect of cell concentration on DNA synthesis stimulated by PMA/A23187 (Table V). There was a relatively linear decline in the magnitude of [^{3}H]Thd incorporation as the B cell density in culture was reduced from 1×10^5 cells/well to concentrations as low as 1.25×10^4 cells/well.

These observations indicate that it is unlikely that factors secreted by non-B cells play a significant role in the magnitude of the B cell responses and suggest that the magnitude of thymidine incorporation that we observe in response to PMA/A23187 is a reflection of DNA synthesis occurring in B cells that have been directly activated by these stimuli.

To establish that [^{3}H]Thd incorporation induced by PMA in combination with A23187 correlated with DNA synthesis, we analyzed propidium iodide-stained B cells on the fluorescence-activated cell sorter to measure DNA content of the cells (Table VI). As has been previously shown, anti-Ig in the presence of BSF-1 is capable of inducing a large fraction of B cells into S phase. In comparison, PMA and A23187 synergistically induced an even greater percentage of B cells to enter DNA synthesis and mitosis.

Dexamethasone suppression of membrane phospholipid hydrolysis. Because our data suggested that dexamethasone was directly affecting B cell activation at a step proximal to activation of PKC, we observed its effect on capping induced by anti-Ig and found that it had no suppressive effect. Thus, greater than 90% of control or dexamethasone-treated B cells that were cultured with anti-Ig antibody demonstrated tight cap formation after 15 min of culture. To determine the effect of dexamethasone on the presumed sequence of events after sIg cross-linking we studied the ability of anti-Ig to cause an increase in the hydrolysis of membrane phosphatidylinositol (Table VII). Incubation of B cells in the presence of

TABLE V

Effect of concentration of cultured B cells on the calcium ionophore and PMA-stimulated B cell activation[a]

	[^{3}H]Thd Incorporation (cpm)				
	2×10^5	1×10^5	5×10^4	2.5×10^4	1.25×10^4
Medium	1,649	518	305	347	273
Anti-IgD	10,497	2,973	920	419	487
PMA	412	191	167	103	250
A23187	882	197	161	296	174
PMA + A23187	37,936	29,124	17,252	6,705	4,290

[a] Small resting B cells that were depleted of NK cells and then size-separated by elutriation were cultured in triplicate at the indicated cell densities in microtiter wells (0.2 ml) for 40 hr in the presence or absence of: anti-IgD, 10 μg/ml; PMA, 10 ng/ml; and/or A23187, 250 ng/ml. Cells were pulsed with 1 μCi of [^{3}H]Thd 18 hr before harvesting. In this experiment the addition of dexamethasone at 10^{-7} M inhibited the anti-IgD-stimulated proliferation from 10,497 to 1,490 cpm but had no significant suppressive effect on the responses stimulated by PMA/A23187 at all cell doses tested. All SD were less than 10% of the mean.

TABLE VI

Induction of B cells into S phase by anti-Ig and by the combination of PMA and A23187[a]

Mitogen	[^{3}H]Thd Incorporation (cpm)	% Cells in S + G_2 + M
Medium	1,983 ± 336	4.4
Anti-IgD	8,611 ± 1,109	23.2
Anti-IgD + BSF-1	36,849 ± 2,059	35.7
PMA + A23187	52,776 ± 4,254	56.1

[a] Small resting B cells (1×10^5 cells/well) were cultured in the presence of: anti-IgD, 10 μg/ml; BSF-1, 10 U/ml; PMA, 10 ng/ml; and/or A23187, 250 ng/ml, for 18 and 36 hr. For measuring cellular DNA synthesis, cells were pulsed with 1 μCi of [^{3}H]Thd 18 hr before harvesting, and the results are expressed as the arithmetic mean of triplicate determinations ± SD. For determination of the percentage of B cells in S + G_2 + M phases of the cell cycle, 2×10^7 cells/2-ml volume were incubated for 36 hr in the presence of mitogens/growth factors as above and then incubated with propidium iodide as described under *Materials and Methods*. The percentage of cells in S + G_2 + M phases was determined by fluorescence-activated cell sorter analysis, measuring the integral of the cells with greater than 2 N DNA content.

TABLE VII

Effect of dexamethasone on accumulation of [^{3}H]inositol phosphates in B cells[a]

	Medium	Dex 6-hr Pulse Followed by 18 hr Incubation	Dex Final 6 hr of Culture
Medium	90 ± 7	96 ± 3	116 ± 10
Anti-IgD			
5 μg/ml	231 ± 19	127 ± 12	196 ± 26
50 μg/ml	339 ± 4	132 ± 9	293 ± 51

[a] Purified B cells (1×10^6) that had been treated with dexamethasone (Dex) 100 nM for 6 hr, washed twice, and then incubated for 18 hr, as well as B cells that were treated with dexamethasone in sustained culture for 6 hr, and B cells in medium alone were [^{3}H]myoinositol-labeled in medium containing Li^+ 10 mM and stimulated with anti-Ig. The accumulation of [^{3}H]inositol phosphates was determined 40 min after stimulation and the results are expressed as the mean cpm of triplicate determinations ± SD.

100 nM dexamethasone for 6 hr before an 18-hr culture period in the presence of medium only and tritiated myoinositol resulted in significant diminution of phosphatidylinositol hydrolysis stimulated by anti-Ig. Similarly, cells that were cultured overnight in the presence of dexamethasone and tritiated myoinositol showed comparable suppression in the anti-Ig-mediated hydrolysis of phosphatidylinositol bisphosphate (PIP_2) (data not shown). The viability of the dexamethasone-treated cells was comparable to that of control cells, and their ability to proliferate in response to PMA/A23187 was not suppressed. On the other hand, dexamethasone administered for only 6 hr before anti-Ig stimulation had no effect on PIP_2 hydrolysis. These observations indicate that a period longer than 6 hr after B cell exposure to dexamethasone is required before its suppressive effects can be observed. This is in agreement with other reports that have demonstrated that dexamethasone mediates its pleiotropic effects on cell activation by inducing several different proteins (21–23).

Dexamethasone suppression of anti-Ig-stimulated calcium translocation. The above finding that dexamethasone suppressed the production of inositol phosphates most probably reflects the suppression of PIP_2 hydrolysis. Alternatively, it was possible that dexamethasone suppressed biosynthetic labeling of PIP_2 by myoinositol. To exclude this possibility, we measured the effects of dexamethasone pretreatment on anti-Ig-mediated calcium metabolism. The initial increase in $[Ca^{2+}]_i$ that is stimulated by anti-Ig antibody is dependent upon the generation of inositol trisphosphate, a product

of PIP_2 hydrolysis. The observation that dexamethasone suppressed PIP_2 hydrolysis suggested that the anti-Ig-stimulated increase in $[Ca^{2+}]_i$ could also be suppressed. B cells were cultured with 100 nM dexamethasone for 7 hr, washed three times and cultured for an additional 12 hr in medium only, and then analyzed by flow cytometry for changes in $[Ca^{2+}]_i$ stimulated by anti-Ig antibody (Fig. 2). The basal $[Ca^{2+}]_i$ was approximately 130 nM and was equivalent in cells pretreated in medium or dexamethasone. Both sets of cells were able to tightly regulate $[Ca^{2+}]_i$ so that less than 5% of the cells had a $[Ca^{2+}]_i$ greater than 215 nM before anti-Ig stimulation. After stimulation, the maximal calcium response occurred approximately 45 sec after the addition of anti-IgD antibody, and the kinetics of this response were not altered in cells pretreated with dexamethasone (Fig. 2). The mean maximal responses were 396 and 290 nM for cells pretreated with medium or dexamethasone, respectively, although the number of cells reaching a $[Ca^{2+}]_i$ greater than 2 SD above the basal mean $[Ca^{2+}]_i$ was nearly equivalent at 88 and 80%, respectively. The decreased magnitude of response seen in dexamethasone-treated cells can be more clearly seen in Figure 3. In this experiment, 93 and 88% of cells pretreated with medium or dexamethasone had a response of greater than 2 SD above the basal $[Ca^{2+}]_i$; however, at the time of the peak response, 44% of cells pretreated with medium had a response greater than 400 nM as compared to only 16% of cells pretreated with dexamethasone. The above findings demonstrated that dexamethasone pretreatment did not alter either the basal $[Ca^{2+}]_i$ nor the histogram of the distribution of B cell $[Ca^{2+}]_i$. By contrast, dexamethasone significantly blunted the mean response to anti-IgD antibody stimulation without significantly altering the percentage of responding cells.

DISCUSSION

The data in this manuscript demonstrate that B cells stimulated by anti-Ig antibodies (both anti-IgD and anti-IgM) are markedly sensitive to the suppressive influence of dexamethasone, whereas B cells stimulated by the combination of PMA and A23187 are resistant. The suppressive effects of dexamethasone appear to reflect its binding to a nuclear receptor which can be inhibited by the specific antagonist RU486 (20). The resistance of PMA/A23187-stimulated B cells occurs at all concentrations at which synergy for the B cell proliferative response exists, and it is therefore very likely that this resistance is not simply a consequence of the potency of the mitogenic stimulus. Furthermore, it is unlikely that PMA/A23187 stimulates small numbers of potentially contaminating non-B cells to secrete factors that may diminish the suppressive effect of dexamethasone on B cells, since PMA/A23187 stimulation of highly purified populations of small resting B cells that were depleted of macrophage and NK cells was equally as resistant to dexamethasone. The finding that dexamethasone inhibits anti-Ig-stimulated entry of B cells into G1 but inhibits neither anti-Ig-mediated cross-linking of sIg nor PMA/A23187-mediated B cell activation suggests that it may affect a pathway of B cell activation that is distal to anti-Ig-induced cross-linking of sIg but proximal to anti-Ig-mediated generation of inositol trisphosphate and diacylglycerol. To explore this hypothesis we studied the effect of dexamethasone on anti-Ig-mediated stimulation of membrane phospholipid metabolism and calcium translocation to determine if its suppressive effects reflected its ability to inhibit the formation of two critical second messengers, diacylglycerol and inositol trisphosphate. The experiments demonstrate that dexamethasone-treated cells show markedly diminished turnover of phosphatidyl inositol as well as reductions in calcium translocation after stimulation with anti-Ig antibody. Since anti-Ig stimulates increases in intracellular calcium both by the generation of inositol trisphosphate and by increasing transmembrane ion fluxes in activated B cells, the residual increases in $[Ca^{2+}]_i$ in stimulated B cells that were dexamethasone-treated may reflect entry via the latter pathway. The findings that dexamethasone inhibits anti-Ig-stimulated B cell size increases as well as inhibiting B cell thymidine incorporation even when added 24 hr after the addition of anti-Ig indicates that this corticosteroid can inhibit both entry of G_0 cells into G_1 as well as entry of G_1 cells into S of the cell cycle. These data are consistent with the idea that continued hydrolysis of phosphatidyl inositol may be critical for the B cell to pass through the cell cycle from G_0 to G_1 as well as from G_1 to S. This would also support previously published findings that culture of cells with anti-Ig antibody for 24 hr, followed by washing and an additional culture period for 24 hr in the presence of growth factors, is not sufficient to stimulate optimal B cell activation, and continued presence of anti-Ig is required (24). Alternatively, these data may indicate that dexamethasone

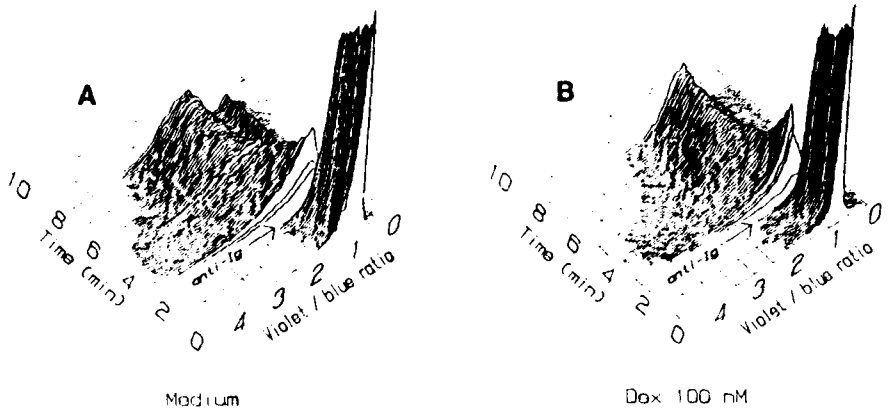

Figure 2. DBA/2 B cells were cultured for 7 hr in dexamethasone (100 nM) or medium, washed twice, and then cultured for an additional 12 hr in medium only. The cells were then loaded with indo-1 and stimulated with anti-IgD antibody (10 μg/ml). The cells were equilibrated at 37°C and analyzed at 400 to 500 cells/sec. The results of real time flow cytometric analysis are shown as isometric plots of indo-1 violet to blue ratio (proportional to $[Ca^{2+}]_i$) vs time vs number of cells. Indo-1 ratios of 1, 2, 3, and 4 are equivalent to $[Ca^{2+}]_i$ of 131, 338, 678, and 1350 nM, respectively. Data are representative of 12 assays from 4 independent experiments.

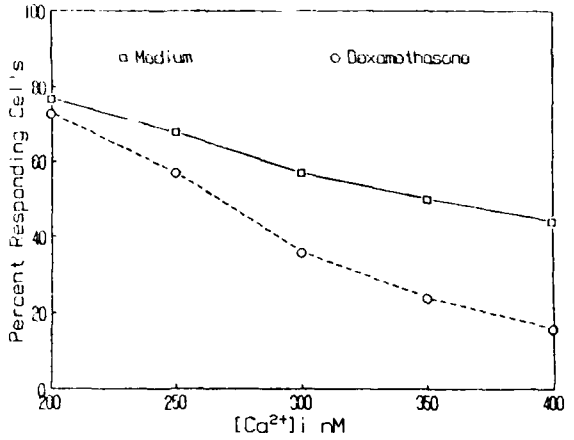

Figure 3. DBA/2 B cells were cultured in medium and dexamethasone, loaded with indo-1, stimulated with anti-IgD antibody, and analyzed by flow cytometry as described in the legend to Figure 2. The mean maximal $[Ca^{2+}]_i$ occurred approximately 45 sec after stimulation and was 388 nM for cells cultured in medium and 253 nM for cells cultured in dexamethasone (not shown). Depicted are the percentages of cells having a calcium response between 200 to 400 nM at the time of the maximal response. The mean basal $[Ca^{2+}]_i$ was 131 nM, and 9 and 7% of cells cultured in medium or dexamethasone, respectively, had $[Ca^{2+}]_i$ greater than 200 nM before anti-Ig stimulation.

can exert its suppressive effect at multiple steps along the pathway of B cell activation.

The suppression of phosphoinositol hydrolysis that is mediated by dexamethasone appears to require a period of time after dexamethasone exposure, since pulsing of B cells for 6 hr with dexamethasone was suppressive only if cells were cultured for an additional 18 hr before addition of mitogen. This is in agreement with the observations of other investigators, studying other cell systems, which indicated that a protein(s) which mediates these suppressive effects may be induced by dexamethasone. An example of such a dexamethasone-inducible protein, lipomodulin, has been shown capable of inhibiting phospholipase C activity, albeit not to the extent that it inhibits phospholipase A_2 (25). Thus, the possibility exists that B cell phospholipase C activity is being suppressed by this dexamethasone-inducible protein. Alternatively, dexamethasone may indirectly affect PIP_2 hydrolysis to inositol trisphosphate and diacylglycerol by inhibiting phospholipase A_2 (26). This enzyme has been shown to account for the majority of arachidonic acid that is derived from the membrane phospholipids and hence for the increased production of prostaglandins in activated cells. The recent observations which demonstrate that the release of arachidonic acid may be required for the control of PIP_2 hydrolysis to inositol trisphosphate and diacylglycerol suggest that a phospholipase A_2-dependent mechanism may account for the suppressive effect of dexamethasone on B cell activation (27, 28).

The susceptibility of B cells to the suppressive effects of dexamethasone when stimulated by LPS is intermediate between that seen on responses stimulated by anti-Ig and PMA/A23187-stimulated B cells. The finding that LPS does not stimulate PIP_2 hydrolysis in B cells nor induce PKC translocation (4, 29, 30) might explain the partial resistance of LPS-stimulated B cell proliferation to dexamethasone-mediated suppression. A possible explanation for the partial inhibition of dexamethasone on LPS-stimulated B cell proliferation may lie in the observation of Rosoff and Cantley (32) who have reported that stimulation of pre-B cells with LPS may partially trigger phosphoinositol metabolism. Thus, dexamethasone may suppress an activation pathway utilized by LPS leading to PKC activation, while not affecting other as yet unidentified pathway(s) of LPS-stimulated cellular activation. An equally plausible alternative possibility is that corticosteroids influence other steps in the B cell activational pathway in addition to phospholipase A_2 and phospholipase C activity. This possibility is supported by other observations[4] that dexamethasone can suppress the ability of BSF-1 to enhance the expression of major histocompatability class II molecules, an event that does not appear to be regulated by phospholipase A_2 or by phospholipase C. It should be stressed, however, that the suppressive effects of dexamethasone appear to be exerted at relatively early events of B cell activation since it does not suppress the latter events stimulated by PMA/A23187 that lead to DNA synthesis.

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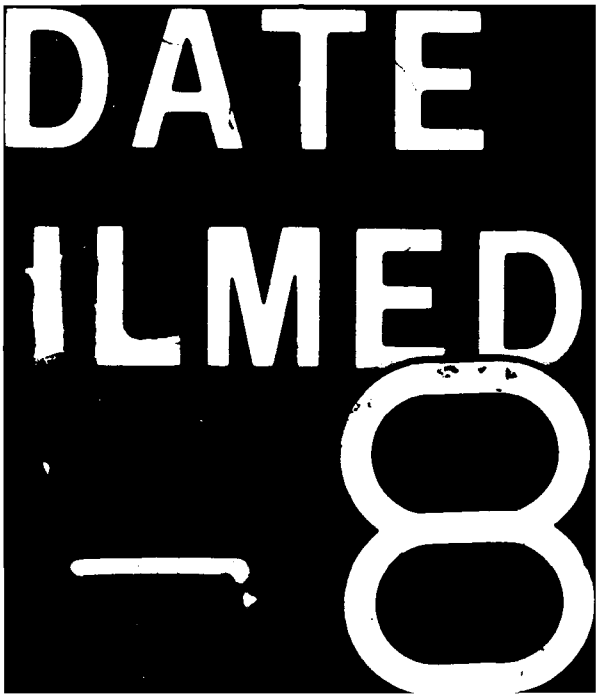
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